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PRINCIPAL INVESTIGATOR: Uma Kamasani, Ph.D.
George C. Prendergast, Ph.D.

CONTRACTING ORGANIZATION: Lankenau Institute for Medical Research
Wynnewood, Pennsylvania 19096

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6. AUTHOR(S)
Uma Kamasani, Ph.D.
George C. Prendergast, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Lankenau Institute for Medical Research
Wynnewood, Pennsylvania 19096

E-Mail: prendergastg@mlhs.org

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Important progress was made this period on the important question of how FTI efficacy is achieved in breast cancer. Clinical trials have revealed that breast cancers respond to FTI but only in a minority of cases. What factors dictate FTI efficacy? Work completed earlier in this project defined roles for RhoB and its downstream effector kinase PRK in mediating growth inhibition by FTI in epithelial cells, including human breast epithelial cells. In this period, progress was made in identifying RhoB effectors that are implicated specifically in FTI-induced apoptosis, a process known to be critical for efficacy. Gene microarray studies identified modules that control cell division, MAPK signaling, and immune response as candidates (in press). We linked two genes that are commonly dysregulated in breast cancer to apoptosis by FTI. Bin1, an adapter-encoding gene implicated in breast cancer suppression, was shown to be essential for FTI-induced apoptosis (in press). Bin1 appears to act downstream of RhoB in apoptosis. Cyclin B1, a key regulator of mitosis, is a critical target for RhoB suppression in FTI-induced apoptosis. We suggest that Bin1 loss and cyclin B1 overexpression, two events that occur commonly in breast cancer cells, may limit FTI efficacy in breast cancer patients.

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Table of Contents

Introduction

We proposed to test the hypothesis that RhoB alteration is responsible for mediating FTI action malignant epithelial cells of the breast. Recent studies suggest that prooncogenic Rho proteins play an important role in driving breast cancer, for example, as in highly aggressive inflammatory breast cancers where overexpression of RhoC is a key oncogenic driver [1,2]. RhoB is an antioncogenic member of the Rho gene family which regulates cellular actin structure, adhesion, motility, proliferation, and survival [3]. RhoB may contribute to the regulation of a signaling cascade that mediates proliferation in response to epidermal growth factor (EGF) [4], a major mitogen for normal and neoplastic breast cells. Moreover, RhoB has been assigned a specialized role in the intracellular trafficking of the EGF receptor [5].

Recent work in our laboratory has identified RhoB as a key target for alteration by farnesyltransferase inhibitors (FTIs), an experimental class of cancer therapeutics that are being tested in clinical trials for breast cancer treatment [6]. In preclinical models, FTIs have displayed relatively unique properties: while largely nontoxic to normal cells they dramatically inhibit the proliferation and/or survival of neoplastically transformed cells. These 'cancer-selective' properties are of significant interest, in part because of they can be traced to molecules other than the molecule that was initially strategized as a target for FTIs, namely Ras [7]. Interestingly, several lines of genetic evidence that have been obtained strongly support a model in which that the antineoplastic properties of FTIs are mediated by a gain-of-function in the antioncogenic RhoB protein [8-12]. Although the main support has derived from mouse models, there is also more recent evidence that RhoB mediates the antineoplastic FTI response in human breast carcinoma cells or RhoC-transformed human mammary epithelial cells [11].

In this research period, we have made progress in addressing a clinically important question that has emerged with the finding that FTIs display efficacy in human breast cancer: what are the factors which dictate efficacy in the minority of breast cancer patients that respond to FTI treatment? The original Aim 1 of the project – to

test whether deletion of the RhoB gene compromised the antitumor response to FTI treatment in mouse models of human breast cancer – has been stymied indefinitely by mouse breeding issues that have arisen. This aim has been put on hold. The original Aim 2 of the project – to explore a role for the RhoB effector kinase PRK in the antineoplastic response to FTI – was addressed substantially in the previous period (published in Oncogene 22: 1124-1134 [2003]). Collaborative work with Sofia Merajver and Kenneth van Golen and their colleagues at the University of Michigan has confirmed the essence of these findings in human breast epithelial cell model (Molec. Cancer Therapeutics, 1: 575-583 [2002] and unpublished results). However, this line of work also suggested that PRK was not involved in FTI-induced apoptosis, a process that is known to be linked to drug efficacy in mice [10]. We therefore sought other effectors of RhoB that were required for FTI-induced apoptosis. Significant progress in this direction, as part of a refocused effort in Aim 2 that was reviewed and approved by the USAMRMC this year, has been made.

Body

Aim 1. This aim included mouse breeding experiments to move the rhoB null allele from a rhoB "knockout" mouse onto various 'oncomouse' models for breast cancer. These included the well-documented mice called mouse mammary tumor virus (MMTV)-neu, MMTV-tumor growth factor-alpha (TGFa), and MMTV-H-ras mice. Last year, we learned that the MMTV-H-Ras strain is unsuited to the study, because of fertility problems in males that were exacerbated by crossing the rhoB null allele into this strain. This year, issues also emerged with regard to breeding and use of the FTI-susceptible MMTV-TGFa strain, which we have found to insufficiently reliable with regard to tumor formation and phenotype. These may relate to modifier issues which result from interbreeding these mice, which are on different strains. In light of the much stronger progress being made in Aim 2, which impact important emerging clinical issues that are directly related to breast cancer treatment, we elected to put further work on this aim on hold.

Aim 2. This year, this Aim was refocused on new developments in work to define the determinants for FTI-induced apoptosis, which is known from mouse studies to be critical for drug efficacy [10]. The original Aim was to assess a possible role for PRK kinase – a key effector kinase for RhoB signaling - in the FTI response. Positive support for the hypothesis that RhoB and PRK mediate growth suppression by FTI in epithelial cells was obtained in the last period [11,12]. However, related studies suggested that PRK might not be important for FTI to trigger apoptosis. For this reason, we initiated efforts to define other RhoB effector molecules that may be important for apoptosis by FTI. Over the past year, these studies matured sufficiently to lead to two publications now in press at Oncogene and Cell Biology & Therapy [13,14]. Preprints of manuscript proofs for these studies are attached (see Appendix).

With regard to the original Aim 2 Tasks, we have essentially completed the work proposed, within the refocus onto new tasks that were approved by the USAMRMC this year. Briefly, in the revised Aim 2 we will determine whether overexpression of the cyclin B1 gene or suppression of the Bin1 gene – two rather common events in breast cancer – is sufficient to render cells FTI resistant.

These latest efforts address the important question of what factors limit FTI efficacy. A comparison of preclinical tests, performed in transgenic mouse models of breast cancer, with clinical tests, in breast cancer patients, indicates that FTIs are far more efficacious in the mouse models than in the patients. Why? Mouse studies argue that the ability of FTIs to kill cells is a key factor in their efficacy [10], a finding that would surprise few. FTI will induce growth inhibition in most human breast cancer cell lines [15], but with the exception of a few lines (e.g. MCF7 [9]), most are not very susceptible to apoptosis. Genetic studies in the mouse prove that the ability of FTI to induce apoptosis depends upon gain-of-function in RhoB. Thus, one logical strategy to define factors that dictate apoptotic susceptibility is to compare the genetic response of cells with different RhoB genotypes to FTI-induced apoptosis. We used a RhoB knockout model and a gene microarray hybridization strategy to focus specifically on events that precede the execution of RhoB-dependent apoptosis (rather than on other aspects of

the FTI response mediated by RhoB gain-of-function, such as growth inhibition. By this approach [14], genes that control cell adhesion and cell shape were represented prominently among upregulated targets of RhoB, as were genes that control signal transduction, vesicle dynamics, transcription, and immunity. Genes that control cell cycle checkpoints and progression through S phase and mitosis were among the major downregulated targets of RhoB. In support of the concept of RhoB as a negative regulator of Ras signaling pathways, the most strongly downregulated gene scored was farnesyl pyrophosphate synthetase, the enzyme that produces the substrate used by FT to farnesylate Ras proteins. Gene clustering revealed modules for MAPK signaling, cell cycle progression, and immune response as proapoptotic targets of RhoB. Recent work has focused on a central cellular regulator, cyclin B1, that was identified in this screen. Further study of the set of this set of genes may yield insights into the dramatic differences in efficacy and apoptotic prowess of most FTIs in human cancers, as compared to transgenic mouse models.

We focused on the relevance of cyclin B1 suppression because of observations from several laboratories that suggest RhoB can affect events in mitosis, including events that impact apoptotic susceptibilities [16]. Cyclin B1 downregulation was observed to occur specifically in cells that were fated to undergo apoptosis after FTI treatment, a process which requires RhoB. To test the hypothesis that downregulation of cyclin B1 was crucial for apoptosis, we enforced its expression in susceptible cells and asked whether this was sufficient to render them resistant. Positive results were obtained: cells overexpressing cyclin B1 were susceptible to FTI-induced growth inhibition, morphological alteration, and actin reorganization, but they were resistant to FTI-induced apoptosis. These findings highlight a specific effect of cyclin B1 on the survival of transformed cells that is distinct from its effects on cell cycle, insofar as cyclin B1-overexpressing cells did not cycle faster than control cells. We confirmed the expectation of the 'gene chip' that RhoB can downregulate the cycli B1 promoter. Preliminary work suggests two further findings. First, studies with RhoB effector domain mutants suggests that RhoB must interact with the downstream actin/microtubule regulator mDia for this activity. mDia is implicated in cytokinesis, consistent with a

connection to mitotic events. Second, it is clear that RhoB affects cyclin B1 at both transcriptional and posttranslational levels; we are examining a possible connection between mDia and Polo kinase, which phosphorylates cyclin B1 protein, as a potential mediator of the posttranslational effects. Current efforts focus on fully defining the signaling linkage between RhoB and cyclin B1 in apoptosis by FTI.

In a second line of work, a serendipitous observation by a member of our laboratory led to the finding that the Bin1 gene is necessary for FTI to trigger apoptosis via RhoB. Bin1 is a tumor suppressor gene that has been previously linked to breast cancer suppression and transformation-selective apoptosis [17-19]. To further develop the hypothesis that Bin1 is a cancer suppression gene, we knocked out this gene in the mouse [20]. Notably, transformed cells that lack Bin1 were found to be resistant to FTI-induced apoptosis [13]. The findings of this study argued that the adapter proteins encoded by the Bin1 gene acted downstream or in parallel to RhoB in cell death signaling. The death defect in *Bin1* null cells was significant insofar it abolished FTI efficacy in tumor xenograft assays. *p53* deletion did not phenocopy the effects of *Bin1* deletion. However, MEFs transformed by SV40 large T antigen+Ras were also resistant to apoptosis by FTI, consistent with other evidence that large T inhibits Bin1-dependent cell death by a p53-independent mechanism. Taken together, the results defined a function for *Bin1* in apoptosis that was conditional on transformation stress, a feature also displayed by RhoB and also by FTI.

The identification of roles for Bin1 and cyclin B1 suppression in apoptosis by FTI are significant to issues surrounding the efficacy of FTI in breast cancer, because cyclin B1 is frequently overexpressed and Bin1 is frequently attenuated in human breast cancers [17]. Thus, these two events may limit susceptibility to FTI treatment in the clinic. While further analysis is needed to substantiate this hypothesis, these two genes represent candidates for response markers in the clinic.

Tasks 2a and 2b to generate and test the *in vitro* FTI response of cell populations where cyclin B1 and Bin1 is manipulated is near complete. Current work focuses on

Tasks 2c and 2d to examine, respectively, the *in vivo* FTI response in tumor allografts and the hypothesized correlation between FTI response and cyclin B1 or Bin1 status in malignant human cell lines.

Key Research Accomplishments

1. Identification of RhoB-dependent gene regulatory events that are associated with FTI-induced apoptosis. The gene microarray strategy and the genes identified are in press at Cell Biology & Therapy (May 2003 issue).
2. Identification of an essential role for the tumor suppressor gene Bin1, downstream or in parallel to RhoB, in FTI-induced apoptosis. This work employed gene 'knockout' cells where the Bin1 gene was ablated. Transformed cells lacking Bin1 were susceptible to FTI-induced growth inhibition and other FTI responses, but they were refractory to FTI-induced apoptosis. This work is in press at Oncogene (June 2003 issue).
3. Identification of the central mitotic regulator Cyclin B1 as an essential target for suppression by RhoB in FTI-induced apoptosis. Work to understand the mechanistic linkage between RhoB and Cyclin B1 is in progress.

Reportable Outcomes

1. A small set of genes have been defined to be correlated with apoptosis by FTI, in particular genes that influence cell adhesion, mitosis, MAPK signaling, and immune response. One unexpected implication to emerge from this study is that FTI may be useful to combine with immunotherapeutic approaches in cancer.
2. Cyclin B1 responsiveness may dictate susceptibility to apoptosis by FTI.
3. Bin1 expression status may dictate susceptibility to apoptosis by FTI.

Conclusions

Two mechanisms of resistance to FTI have been defined, both of which occur commonly in human breast cancers. These events may limit FTI efficacy in many

breast cancer patients. The identification of cyclin B1, Bin1, and other candidate genes that are important for the apoptotic response to FTI may promote the useful application of FTI in the clinic to treat breast cancers, by identifying patients that are mostly likely to respond to drug.

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Appendices

Original Research Reports (preprints)

DuHadaway, J.B., Du, W., Liu, A.-X., Baker, J., Donover, P.S., Sharp, D.M., Muller, A.J., and Prendergast, G.C. (2003). Transformation selective apoptotic program triggered by farnesyltransferase inhibitors requires Bin1. Oncogene **22**, in press.

Kamasani, U., Liu, A.-X., and Prendergast, G.C. Genetic response to farnesyltransferase inhibitors: proapoptotic targets of RhoB. Cancer Biol. Therapy **2**, in press.

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Research Paper

Genetic Response to Farnesyltransferase Inhibitors

Proapoptotic Targets of RhoB

Uma Kamasani[1,†]
Ai-xue Liu[2,†,#]
George C. Prendergast[1,3*]

[1]The Lankenau Institute for Medical Research; Wynnewood, Pennsylvania USA

[2]The Wistar Institute; Philadelphia, Pennsylvania USA

[3]Department of Pathology, Anatomy, and Cell Biology; Jefferson Medical School; Thomas Jefferson University; Philadelphia, Pennsylvania USA

[†]These authors contributed equally to this study and should be considered co-first authors.

[#]Current address: GlaxoSmithKline, Inc., King of Prussia, Pennsylvania USA

*Correspondence to: George C. Prendergast: Lankenau Institute for Medical Research; 100 Lancaster Avenue;Wynnewood , Pennsylvania 19096 USA; Tel.: 610.645.8475; Fax: 610.645.8091; Email: prendergastg@mlhs.org. Website: www.limr.or

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KEY WORDS

Ras. Apoptosis, Rho

ABSTRACT

Knockout mouse studies have established that the transformation-selective death program triggered by farnesyltransferase inhibitor (FTI) requires a gain-of-function in the stress-regulated small GTPase RhoB. To gain insight into this death program, we compared the genetic response of cells with different RhoB genotypes to FTI treatment. The microarray hybridization strategy we employed focused specifically on events preceding the execution of RhoB-dependent apoptosis, which is crucial for effective antineoplastic responses in mouse, rather than on other aspects of the FTI response mediated by RhoB gain-of-function (e.g., growth inhibition). Genes that control cell adhesion and cell shape were represented prominently among upregulated targets, as were genes that control signal transduction, vesicle dynamics, transcription, and immunity. Genes that control cell cycle checkpoints and progression through S phase and mitosis were among the major downregulated targets. In support of the concept of RhoB as a negative regulator of Ras signaling pathways, the most strongly downregulated gene scored was farnesyl pyrophosphate synthetase, the enzyme that produces the substrate used by FT to farnesylate Ras proteins. Gene clustering revealed modules for MAPK signaling, cell cycle progression, and immune response as proapoptotic targets of RhoB. This report identifies genes that pertain to the transformation-selective apoptotic program triggered by FTI. Further study of this program may yield insights into the dramatic differences in efficacy and apoptotic prowess of most FTIs in human cancers, versus transgenic mouse models.

INTRODUCTION

Apoptosis is thought to play a major role in cancer suppression and therapeutic response. Of particular interest are the death processes in cells harboring neoplastic lesions. While great progress has been made in identifying the basic mechanisms of apoptosis, which are critical to development and normal physiology, much less progress has been made in defining the precise mechanisms that stanch cancer and that are selectively inactivated during cancer progression and the development of therapeutic resistance. Such mechanisms are of great interest, since they may help unravel the precise relationship between apoptosis and cancer pathophysiology, as well as help identify disease-specific therapeutic strategies.

We have investigated the transformation-selective properties of some farnesyltransferase inhibitors (FTIs) as a way to gain insight into cancer-specific apoptosis. FTIs are a class of experimental chemotherapeutic agents that are potent inducers of p53-independent apoptosis in oncogene-transformed cells, but that in most cases have little effect on the survival of untransformed cells (reviewed in refs. 1 and 2). FTIs were developed initially as a strategy to attack the farnesylation requirement of oncogenic Ras in human cancers. Preclinical tests have established the antineoplastic properties of FTIs in various model systems, including proapoptotic effects; based on these tests FTIs have moved to clinical trials. However, the antineoplastic properties of FTIs can be traced beyond Ras targeting. Moreover, while some have been encouraged by the results of clinical testing, it seems that FTIs lack the robust antitumor effects they display in various preclinical models,[3,4] perhaps explaining why such a small number of compounds remain in clinical trials despite the enormous effort this project engendered in the 1990s. In preclinical models, FTI efficacy has been linked to apoptotic susceptibility, thus, it is logical to link the lack of clinical efficacy to apoptotic deficiencies in human cancer cells. How is transformation-selective apoptosis possible in preclinical models? How is this intriguing and desirable aspect of FTI biology defeated in human cancers? Addressing these questions may define appropriate clinical application, improve therapeutic response, and give insights in how cancer progression selects against the cancer-selective apoptotic program triggered by FTI.

Our studies of the FTI response in rodent model systems led to the development of a model featuring the small GTPase RhoB as a critical gain-of-function target.[5] A genetic proof of the essential role of RhoB in apoptosis by FTI has been established by studies in a knockout mouse model system.[6] Work in this model also revealed that, while RhoB is dispensable for physiological apoptosis during development,[7] it is also crucial for apoptosis triggered in transformed cells by clinically effective therapeutics such as DNA damaging agents and paclitaxel.[8]

To gain insight into the mechanisms through which FTI triggers apoptosis through RhoB, we compared the genetic response of transformed mouse embryo cells with different RhoB genotypes to treatment with the potent and specific FTI L-744,832 (ref. 9). FTIs target a broad spectrum of farnesylated proteins in cells, so their biological and epigenetic effects are quite broad and complex. However, by focusing specifically on the genes that respond under conditions of RhoB-dependent apoptosis, we defined only those gene regulatory events that are coupled specifically to the transformation-selective program of cell death triggered by FTI, a program that is critical for in vivo antineoplastic efficacy.[6] This study identifies regulators of chromatin, immunity, and mitosis as previously uncharacterized targets of RhoB regulation in cancer cells that are fated to undergo apoptosis.

MATERIALS AND METHODS

Cells and Culture Conditions. $ER^{+/-}$ MEFs and $ER^{-/-}$ MEFs are E1a/Ras-transformed mouse embryo fibroblasts derived from *rhoB*$^{+/-}$ or *rhoB*$^{-/-}$ animals.[6] Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS; Hyclone). Separate cultures of asynchronously growing cells were fed with medium containing 10% or 0.1% FBS for 16 hr and then treated with 10 μM FTI L-744,832 or vehicle as described previously.[6] Twelve hr after drug addition cells were harvested for preparation for RNA. Parallel cultures of cells treated under similar conditions were monitored to confirm that only cells carried in 0.1% FBS + FTI underwent apoptosis as expected at later times, as described previously.[6] Time course experiments designed similarly were performed to obtain protein extracts for Western analysis.

Microarray Analysis. MA-01 mouse cDNA filter arrays were purchased from The Wistar Institute Genomics Facility (http://www.wistar.upenn.edu/genomics/). Three 2.5 x 7.5 cm nylon filters carrying a total of 6600 probes for 1680 individual genes were used for hybridization; samples were hybridized as a single batch on sequentially printed arrays. All arrays used in the study were printed from the same PCR preparations of sequence verified clones purchased from Research Genetics. Reproducibility studies showed a >90% correlation between samples hybridized in triplicate. Complete protocols for array hybridization can be accessed at:

http://www.wistar.upenn. edu/genomics/

Briefly, total RNA was isolated from ER MEFs using Tri-reagent (Molecular Research Center) according to the manufacturer's instructions. The total RNA target (5 μg) was labeled with ^{33}P (3000–5000 Ci/mM) (Amersham Pharmacia Biotech) using reverse transcriptase. Hybridization of filters was performed in 2.5 ml of Micro-Hyb (Research Genetics) at 42°C for 18 h. Filters were exposed to a PhosphorImager screen for 4 days, scanned at 50 micron resolution on a Storm PhosphorImager, and visualized using ImageQuant (Molecular Dynamics). The data for each array was analyzed with ArrayVision (Imaging Research), using the median pixel for each spot and local background correction. Data was exported to Excel for further analysis. Expression values for each array were normalized by the background corrected signal median spot of the array. Expression values were transformed to corresponding z-scores for clustering. T-tests, frequency analysis, and permutations were done using Excel and Visual Basic. Dynamic range of signals was, on average, 10–20,000 (normalized median density of 0.15 to 3000). The detection limit for these conditions and arrays calibrated by quantitative PCR with a plasmid standard is approximately 0.03 molecules per cell.

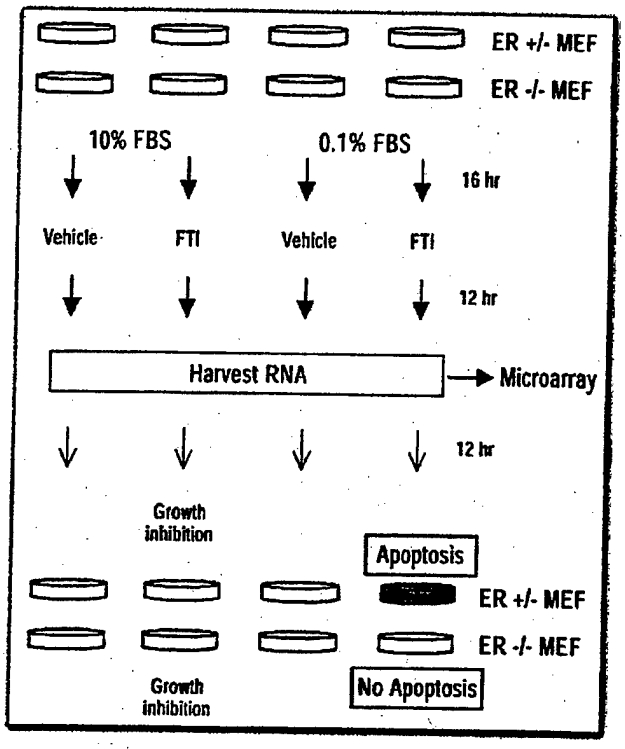

Figure 1. The design of the cell experiment from which RNAs were derived for microarray hybridization is shown. $ER^{-/-}$ MEFs are susceptible to growth inhibition and morphological reversion by FTI, but are refractory to actin reorganization and apoptosis by FTI like $ER^{+/-}$ MEFs. Apoptosis is only triggered in $ER^{+/-}$ cells under conditions of serum deprivation. RNA was harvested from cells 12 hr after FTI treatment, preceding the execution phase of apoptosis by 6–12 hr.[6]

Western Analysis. Cell extracts was prepared by lysing cells in NP40 lysis buffer supplemented with protease inhibitors. Western analysis of proteins in cell extracts was performed as described[10] using antibodies to cyclin B1 and cdc6 (Santa Cruz Biotechnology).

RESULTS AND DISCUSSION

Isolation of RNA from Cells Committed to Undergo FTI-Induced Apoptosis. A well-characterized pair of E1a/ras-transformed mouse embryo fibroblast populations that are heterozygous or nullizygous for RhoB, termed $ER^{+/-}$ or $ER^{-/-}$ cells,[6-8] were used to compare the genetic response to the potent and specific FTI L-744,832 (ref. 9) at early times after drug treatment. Previous work has established that $ER^{+/-}$ cells are highly susceptible to FTI-induced apoptosis whereas $ER^{-/-}$ are refractory but still sensitive to growth inhibition.[6] Serum growth factors and substratum attachment prevent FTI-induced apoptosis, which is induced if growth factors or attachment are deprived.[11-13] Apoptosis induced in $ER^{+/-}$ MEFs under conditions of serum deprivation is initiated starting at 18–24 hr after drug treatment.[6] Figure 1 presents the design of the experiment performed in the current study. Briefly, $ER^{+/-}$ and $ER^{-/-}$ cells were carried for 16 hr in normal or serum-deprived media, treated with 10 μM FTI or vehicle for 12 hr, and then harvested for preparation of total RNA. Parallel cultures were monitored to confirm that $ER^{+/-}$ cells cultured under low serum conditions committed to undergo apoptosis at later times (Fig. 1, bottom), as measured by cleavage of the caspase-3 substrate PARP (data not shown).

Functional Categories of RhoB Target Genes in Cells Committed to Undergo FTI-Induced Apoptosis. Unpublished studies suggest that FTI regulates a very large number of genes, as would be anticipated since the

Table 1 **FUNCTIONAL CATEGORIES OF RHOB TARGET GENES IN FTI-INDUCED APOPTOSIS**

Function	Gene	Activated (+) or Repressed (-)	Fold
Cell adhesion	Procollagen III α1	+	11.88
Extracellular matrix	Tenascin C	+	5.69
Cytoskeleton	Procollagen IV α1	+	5.16
	HIC-5	+	5.12
	Collapsin response mediator protein 1	+	4.64
	LOX2	+	4.23
	SDR1	+	4.00
	Osteopontin	+	3.31
	EXT1	+	3.27
	Cofilin	+	3.21
	CD151	+	3.04
	Myosin light chain-1	+	2.93
	α-actinin 3	+	2.84
	Islr	+	2.70
	α-actinin 1	+	2.68
	Rac1	+	2.62
Cell cycle	Mad2	+	3.74
	Cdc6	-	-3.66
	Cyclin B1	-	-3.40
	Mcm4	-	-3.19
	Rabkinesin-6	-	-2.31
	Cdc20	-	-2.12
Signal Transduction	MAP kinase phosphatase 1 (MKP1)	+	4.47
	Protein phosphatase PP2A α isoform	+	4.24
	B-Raf	+	3.20
	Neuronal calcium sensor-1 (NCS-1)	+	3.10
	Protein tyrosine phosphatase, receptor type A	+	2.50
	FKBP-1α	-	-2.75
	K-Ras	-	-2.50
Vesicle fusion	Rab6	+	2.94
Endocytosis	ATP2α2	+	2.93
Membrane Dynamics	Unc-18 homolog	+	2.92
	Annexin V	+	2.89
	Disabled homolog 2 (Dab2)	+	2.69
	Acylphosphatase	-	-3.11
	Synaptotagmin-4	-	-2.90
Lipid synthesis/metabolism	Apolipoprotein A-IV	+	3.02
	Choline/Ethanolamine kinase	+	2.55
	Farnesyl pyrophosphate synthetase	-	-11.93
Transcription	HOX-B2	+	3.99
Chromatin dynamics	Mitochondrial transcription factor A	+	3.51
	PBX1	+	2.98
	Cysteine-rich protein 2 (LIM domain protein)	+	2.75
	Myc	+	2.60
	NeuroD3	+	2.56
	CHD-1	-	-6.55
	Maf	-	-2.95
	Histones H1, H2A, H3	-	2.94-2.68
	STAT1	-	-2.75
Translation	eIF-4E	+	3.01
	EF1α-1	-	-2.30
Immune Response	Lipocortin	+	5.67
	Lymphotoxin B	+	4.24
	IL-12	+	2.96
	M-CSF	+	2.96
	Pre-B-cell colony enhancing factor +	-	-2.87
Protein Stability	Proteasome α7/C8 subunit	+	3.16
	Hsp70-2	+	3.08
Energy Metabolism	NADH-ubiquinone oxidoreductase subunit B14.5B	+	3.91

drug affects the prenylation of as many as 0.5% of all cellular proteins.[14] We exploited the RhoB knockout system to focus on only those FTI-regulated genes that are associated with activation of the transformation-selective apoptotic program. RhoB-dependent and RhoB-independent events that occur in serum deprived cells or FTI-treated cells are not of interest. We are only interested in events that occur in serum deprived and FTI-treated cells—since that condition specifically elicits transformation-selective apoptosis—and only if those events are RhoB-dependent—since that condition is essential for apoptosis.[6] In our experimental design (see Fig. 1), the genetic response of $ER^{+/-}$ and $ER^{-/-}$ cells was compared under conditions of serum deprivation + FTI at a time that in $ER^{+/-}$ cells precedes the effector stage of apoptosis by at least 6 hr.[6] This design selects against the many genes that are regulated by serum deprival alone or FTI alone (by either RhoB-dependent or RhoB-independent mechanisms). In short, the genes identified represent a comparison of the genetic response in the far right dishes noted in Figure 1. By exploiting the RhoB knockout system in this manner, we defined a small subset of genes that are specifically relevant to apoptosis and therapeutic response, among the far larger number of genes that are generally regulated by FTI in cells.

Analysis of the microarray hybridization results revealed that 59/1680 genes (3.51%) were differentially activated and 19/1680 genes (1.13%) were differentially repressed in $ER^{+/-}$ cells as compared to $ER^{-/-}$ cells at an arbitrary conservative cutoff of 2.50-fold change. Decreasing the cutoff stringency to a less conserative value of 2.0-fold change added 68 genes to the number of differentially activated targets (to 7.50% of genes surveyed), but only 4 genes to the number of differentially repressed targets (to 1.37% of genes surveyed). Thus, by 12 hr after FTI addition, a larger fraction of genes were activated, as compared to repressed, in cells committed to undergo RhoB-dependent apoptosis triggered by FTI.

Proapoptotic RhoB target genes identified in this way fell into several functional categories as presented in Table 1. Among upregulated targets, genes that control cell adhesion and cell shape were represented prominently, including components of the extracellular matrix and actin cytoskeleton. This functional category included a majority of the most strongly activated targets. Among other upregulated targets were key regulators of signal transduction, vesicle trafficking, membrane dynamics, transcription, and immunity. Links of RhoB to immunity are novel. However, links to the other functional categories are consistent with accepted functions of Rho proteins and RhoB itself, which has a

Table 2 **Genes Activated by FTI in Cells Fated to Undergo RhoB-Dependent Apoptosis**

Gene	Function	Fold Increase	Comment
Procollagen, type III alpha 1	Extracellular matrix	11.88	Induction similar to collagen I_2 implicated in FTI-induced reversion[16]
ESTs weakly similar to Agrin Precursor	Unknown	8.86	Agrin is a heparin sulfate proteoglycan that binds tenascin
Tenascin C	Extracellular matrix	5.69	Extracellular matrix protein that modulates cell adhesion via Rho pathways[17]; implicated in tissue remodeling and angiogenesis; promotes FAK phosphorylation and cell migration
Lipocortin 1 (Annexin I)	Immune response	5.67	Implicated in anti-inflammation
Procollagen, type IV alpha 1	Extracellular matrix	5.16	Induction similar to collagen Ia2 implicated in FTI-induced reversion[16]
HIC-5	Cell adhesion	5.12	Focal adhesion-associated LIM domain protein recruited to nucleus 19 and linked to cell senescence.[38]
ESTs weakly similar to zinc finger type transcription factor MZF-3	Unknown	5.03	
Collapsin response mediator protein 1	Cell adhesion	4.64	Suppressor of tumor invasion[21]
MAP kinase phosphatase 1 (MKP1)	Signal transduction	4.47	Downregulates MAPK activity
ESTs moderately similar Endothelial Actin-Binding Protein	Unknown	4.41	
Lymphotoxin B	Immune response	4.24	TNF-related death receptor ligand
Protein phosphatase PP2A, 55 kD regulatory subunit, alpha isoform	Signal transduction	4.24	
Lysyl oxidase-2 (LOX2)	Extracellular matrix	4.23	
SDR1	Cell adhesion	4.00	IgG superfamily receptor
Homeobox protein HOX-B2	Transcription	3.99	
NADH-ubiquinone oxidoreductase subunit B14.5B	Energy Metabolism	3.91	Mitochondrial enzyme
MAD homolog 2	Cell cycle degradation of cyclin B1	3.74	Spindle checkpoint protein; regulates APC-dependent
ESTs moderately similar to Reticulocalbin Precursor	Unknown	3.70	
Transcription factor A, mitochondrial	Transcription	3.51	
ESTs highly similar to Hypothetical 28.9 kD protein C28H8.6 (*C. elegans*)	Unknown	3.40	
ESTs highly similar to Hypothetical 64.5 kD protein ZK652.9 (*C. elegans*)	Unknown	3.32	
PNG protein	Unknown	3.32	
Secreted phosphoprotein-1 (Osteopontin)	Extracellular matrix	3.31	Cytokine that binds integrins; implicated in cell migration and metastasis
Exostoses (multiple) 1 (EXT1)	Extracellular matrix	3.27	Heparin sulfate biosynthesis
Destrin (Cofilin)	Cytoskeleton	3.21	Actin severing protein
B-raf transforming gene	Signal transduction	3.20	Oncogene
Proteasome alpha7/C8 subunit	Protein stability	3.16	
Neuronal calcium sensor-1 (NCS-1)	Signal transduction	3.10	
Hsp70-2	Protein stability	3.08	
CD151 antigen	Cell adhesion	3.04	Integrin-binding tetraspanin protein
Apolipoprotein A-IV	Lipid metabolism	3.02	Lipoprotein component
ESTs moderately similar to Eukaryotic peptide chain release factor GTP-binding subunit (*S. cerevisiae*)	Unknown	3.01	

Continued on next page.

Table 2, cont. **Genes Activated by FTI in Cells Fated to Undergo RhoB-Dependent Apoptosis**

Gene	Function	Fold increase	Comment
Eukaryotic translation initiation factor 4E (eIF4E)	Translation	3.01	Oncogene
ESTs moderately similar to Biotin Carboxylase (Anabaena pcc7120)	Unknown	3.01	
Pbx1 (pre-B-cell leukemia transcription factor 1)	Transcription	2.98	
IL-12 p40	Immune response	2.96	
Colony stimulating factor, macrophage (M-CSF)	Immune response	2.96	
Neuronal protein 3.1	Unknown	2.95	
Rab6	Vesicle fusion	2.94	
Myosin Light Chain 1	Cytoskeleton	2.93	Skeletal muscle isoform
ATP2a2/ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	Membrane dynamics	2.93	Cardiac sarcoplasm reticulum ATPase
unc-18 homolog	Vesicle fusion	2.92	Interacts with exocytotic syntaxin complex
Annexin V	Membrane dynamics	2.89	Binds phosphatidylserine
ESTs highly similar to 34.7 kD protein in SHM1-MRPL37 intergenic region (*S. cerevisiae*)	Unknown	2.84	
ESTs highly similar to 66.5 kD protein in ADE12-RAP1 intergenic region (*S. cerevisiae*)	Unknown	2.84	
α-actinin 3 (Actn3)	Cytoskeleton	2.84	Crosslinks actin filaments
Cysteine-rich protein 2	Transcription	2.75	LIM domain protein
ESTs weakly similar to Hypothtical 39.2 kD protein in EGT2-KRE1 intergenic region (*S. cerevisiae*)	Unknown	2.71	
Isk/Immunoglobulin superfamily containing leucine-rich repeat	Cell Adhesion (?)	2.70	Ig superfamily protein
Dab2/Disabled homolog 2	Endocytosis (?)	2.69	Putative tumor suppressor; implicated in clathrin-mediated endocytosis
α-actinin 1, cytoskeletal isoform	Cytoskeleton	2.68	Actin fiber crosslinking
DNA segment, Chr 15, Wayne State University 77, expressed	Unknown	2.63	
Rac1	Cytoskeleton	2.62	
Myc oncogene	Transcription	2.60	
Mitochondrial Lon Protease Homolog Precursor	Unknown	2.60	Mitochondrial matrix protease implicated in aging
Neurogenic Differentiation 3 (neuroD3)	Transcription	2.56	
Choline/Ethanolamine Kinase	Lipid synthesis	2.55	Catalyzes first step in phosphatidylethanolamine synthesis
Protein Tyrosine Phosphatase, Receptor Type A	Signal transduction	2.50	

specialized physiological function in intracellular vesicle trafficking.[15] Among the downregulated targets, genes that control cell cycle progression were represented prominently. At a reduced cutoff of 2.0-fold change, 2/4 of the additional targets were also cell cycle regulators, such that 6/23 (26%) of the repressed genes fell into this category. Notably, the most strongly downregulated gene that was scored was farnesyl pyrophosphate synthetase, the enzyme that synthesizes the isoprenyl substrate used by FT to catalyze protein farnesylation. Thus, a major target for RhoB repression in cells committed to undergo FTI-induced apoptosis was an enzyme essential for farnesylation of Ras and other cellular proteins. Among other downregulated targets were K-Ras and regulators of transcription and chromatin. In summary, although fewer genes were downregulated, the functional catagories of these genes were at least as intriguting as activated targets with regard to how RhoB might regulate transformation-selective apoptosis by FTI.

Genes Targeted by RhoB in Cells Fated to Undergo FTI-Induced Apoptosis

Cell Adhesion and Cell Shape. The identification of procollagen III and IV alpha-1 subunit genes as RhoB targets paralleled a previous report that procollagen I alpha-2 subunit is upregulated by FTI and essential for FTI-induced reversion of Ras-transformed Rat1 fibroblasts.[16] Tenascin-C,

Table 3 **GENES REPRESSED BY FTI IN CELLS FATED TO UNDERGO RHOB-DEPENDENT APOPTOSIS**

Gene	Function	Fold decrease	Comment
Farnesyl pyrophosphate synthetase	Lipid synthesis	-11.93	Generates activated pool of farnesyl isoprenoid used for protein farnesylation by FT
Chromodomain helicase DNA binding protein-1 (CHD-1)	Chromatin dynamics	-6.55	ATP-dependent chromatin modification
ESTs weakly similar to Germ Cell-Less Protein (nuclear pore associated)	Unknown	-5.38	
ESTs weakly similar to Hypothetical Protein in PRE3-SAG1 Intergenic Region	Unknown	-4.00	
Cdc6	Cell cycle	-3.66	Regulates MCM complex required to license DNA replication in S phase; also functions in G2 phase to prevent the onset of mitosis
Cyclin B1	Cell cycle	-3.40	Regulates mitotic kinase CDK1
Minichromosome maintenance deficient-4 homolog (MCM4)	Cell cycle	-3.19	Component of MCM complex required to license DNA replication in S phase; binds DNA in M phase (anaphase) via a mechanism that requires Cdc6
Acylphosphatase, muscle type isoform	Membrane dynamics	-3.11	
v-Maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	Transcription	-2.95	Possible regulators of p53 transcription
Histone gene complex 1	Chromatin dynamics	-2.94	
Synaptotagmin-4	Vesicle fusion	-2.90	Involved in Ca++ mediated exocytosis
Pre-B-cell colony-enhancing factor	Immune response	-2.87	
Fructose bisphosphate aldolase C	Glycolysis	-2.86	Binds IP3
Signal transducer and activator of transcription 1 (STAT1)	Transcription	-2.75	
FK506 binding protein 1a	Signal transduction	-2.75	
H3 histone, family 2	Chromatin dynamics	-2.75	
H2A.1 histone	Chromatin dynamics	-2.68	
T-complex protein 1, alpha subunit B	Unknown	-2.55	
Ki-Ras cellular oncogene	Signal transduction	-2.50	
Rabkinesin-6	Cell cycle	-2.31	Mitotic function in anaphase or cytokinesis
Elongation factor 1-alpha 1 (EF1α1)	Translation	-2.30	Bundles actin filaments
CDC20	Cell cycle	-2.12	Spindle checkpoint protein; regulates APC-dependent degradation of cyclin B1 in mitosis
ESTs weakly similar to Neurogranin (CaM interacting)	Unknown	-2.12	

another extracellular matrix protein, limits matrix deposition, modulates cell adhesion, regulates phosphorylation of the integrin-associated tyrosine kinase Fak, and suppresses RhoA activity.[17,18] Several other extracellular matrix components are included in this subgroup. Cell adhesion and actin regulatory proteins are also represented, including two Ig superfamily proteins, the integrin-binding tetraspanin CD151, α-actinin, cofilin, myosin light chain-1 (MLC1), Rac1, the Fak-binding LIM domain protein HIC-5, and the suppressor of tumor invasion collapsin response mediator protein-1.[19-21] Many of these proteins have been linked to cancer suppression. In summary, a major category of RhoB regulated targets in cells committed to undergo apoptosis relate to cell adhesion and cell shape.

Cell Cycle Regulation. Genes in this category suggest the regulation of two cell cycle regulatory modules by RhoB, one which regulates the DNA licensing complex in S phase, and a second which regulates the spindle checkpoint in mitosis. Cdc6 is required to regulate the activity of the MCM complex that licenses firing of DNA replication origins in S phase. The MCM component Mcm4 was also identified as an upregulated target. Cdc6 also has a checkpoint role that prevents mitosis before DNA replication is complete.[22] Interestingly, Cdc6 is rapidly destroyed by a p53-independent pathway during early stages of apoptosis triggered by DNA damage, or by a caspase-dependent pathway in cells triggered to undergo apoptosis by tumor necrosis factor.[23] The destruction of Cdc6 may be a primordial programmed death response that uncouples DNA replication from the cell division cycle, which is reinforced in metazoans by the evolution of caspases and p53.[23] The p53-independent aspects of FTI apoptosis via RhoB dovetail with a possible role for Cdc6 downregualtion in apoptosis.

A second cell cycle regulatory module downregulated by RhoB is comprised of Mad2, Cdc20, and cyclin B1. Mad2 and Cdc20 regulate the spindle checkpoint via control of the anaphase-promoting complex.[24] Cyclin B1 is targeted by this complex. Further focus on this mitotic regulatory module is suggested by another downregulated target in this category, rabkinesin-6, which regulates cytokinesis.[25] An integration with Cdc6 and

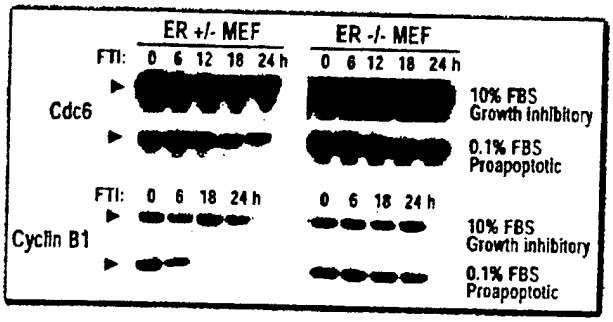

Figure 2. Selective downregulation of Cyclin B1 and Cdc6 proteins by RhoB in cells fated to undergo FTI-induced apoptosis. Western analysis confirmed downregulation of cyclin B1 and cdc6 at steady-state protein levels.

Mcm4 regulation is possible, as both these factors also act in G_2/M phases.[22,26] By Western analysis, we confirmed selective downregulation of cyclin B1 and Cdc6 in FTI-treated cells that are fated to undergo RhoB-dependent apoptosis (see Fig. 2). Consistent with the microarray design, downregulation of these proteins did not occur under conditions of FTI-induced growth inhibition, but only under proapoptotic conditions. Taken together, these observations reinforce the theme that RhoB targets two central cell cycle regulatory modules that control progression through S phase and mitosis.

Signal Transduction. Genes in this category suggest the regulation of a MAPK signaling module that is comprised of K-Ras, B-Raf, the MAPK phosphatase MKP-1, and a protein phosphatase PP2Acα isoform. Although B-Raf is upregulated, the downregulation of K-Ras and the robust upregulation of the inhibitory phosphatases suggests that this MAPK module is functionally downregulated, possibly promoting a loss of cell viability. Interestingly, several studies have linked PP2A to cancer-selective apoptosis.[27] This identification of this target is intriguing relative to the transformation-selective and RhoB-dependent death properties of FTI.

Vesicle Trafficking, Membrane Dynamics, and Lipid Synthesis/Metabolism. Genes in this category reinforce the function of RhoB in intracellular vesicle trafficking, although they do not immediate suggest modulation of a distinct functional module. Suppression of farnesyl pyrophosphate synthetase, the most strongly regulated target in this category, may strength the restriction in protein farnesylation by FTI. However, why this target should respond preferentially in cells fated to undergo apoptosis is unclear.

Transcription and Chromatin Dynamics. Upregulated genes included the proapoptotic oncoprotein Myc. Although the significance of this observation is unclear, a recent study has presented evidence that Bin1, a Myc-interacting adapter protein implicated in apoptosis,[28-31] has an essential role in FTI-induced apoptosis.[32] Downregulated genes included CHD-1, a modifier of chromatin organization that may impact cancer gene expression.[33] Common downregulation of histones 1, 2A, and 3 further suggests a role for chromatin modulation in apoptosis, perhaps as a prelude to DNA degradation. Other downregulated targets encode STAT1, a member of the STAT gene family that can compromise cell survival,[34] and Maf, a transcription factor that can activate the mouse p53 promoter;[35] downregulation of these targets seems counterintuitive in this setting, however.

Immunity. Genes in this category suggest the regulation of an proapoptotic module that operates in vivo. Several important regulators of inflammation were identified as upregulated targets. Lipocortin (annexin-1), the most strongly regulated target in this category, is thought to have an anti-inflammatory role[36] which in this context may promote a 'silent' apoptosis by FTI in vivo. Lymphotoxin B is a death receptor ligand that is related to the tumor necrosis factor (TNF) family. Upregulation of this target could conceivably promote apoptosis via autocrine or paracrine mechanisms. IL-12 and M-CSF promote the development of cytotoxic T cells and monocytes, two classes of cells that promote cancer suppression and apoptosis in vivo. The identification of this novel proapoptotic module targeted by RhoB suggests the possibility of combining FTI with immune-based therapies to promote apoptosis and cancer rejection.

Other Targets. Translation factors eIF-4E and EF1α-1 were identified as upregulated or downregulated targets respectively. The meaning of the opposite regulation of these prooncogenic targets is unclear. Two regulators of protein stability, a proteosome subunit and a chaperone, were identified as upregulated targets. Hsp70 can inhibit apoptosis, possibly through sequestration of the mitochrondrial apoptosis inducing factor AIF,[37] but its upregulation in cells destined to undergo apoptosis is counterintutive.

SUMMARY

This study identifies three potential regulatory modules as candidates for RhoB downregulation in cells that fated to undergo transformation-selective apoptosis by FTI. A MAPK signaling module that includes K-Ras and B-Raf that promotes transformation and cell survival may be targeted by RhoB. Similarly, RhoB may target two cell cycle regulatory modules that are central players in S phase and mitotic progression. Lastly, the identification of an immune module that promotes 'silent' apoptosis may open new vistas on the basis for RhoB-dependent apoptosis in vivo. Further investigations are required to examine the hypotheses prompted by this study, which may lead to insights into why FTIs are strongly apoptotic and efficacious in preclinical models, but much less so in clinical settings.

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Transformation-selective apoptotic program triggered by farnesyl transferase inhibitors requires Bin1.

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ORIGINAL PAPER

Role of Bin1 in apoptosis is contingent upon transformation stress

James B. DuHadaway[1], Wei Du[2], Scott Donover[1], Judith Baker[2,5], Ai-xue Liu[3,6], Diane M Sharp[2], Alexander J Muller[1] and George C Prendergast*[,1,4]

[1]*Lankenau Institute for Medical Research, Wynnewood, PA, USA;* [2]*Cancer Research Group, The DuPont Pharmaceuticals Company, Glenolden, PA, USA;* [3]*The Wistar Institute, Philadelphia, PA, USA;* [4]*Department of Pathology, Anatomy, and Cell Biology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA, USA*

FTI; farnesyltransferase

Neoplastic transformation sensitizes many cells to apoptosis. This phenomenon may underlie the therapeutic benefit of many anticancer drugs, but its molecular basis is poorly understood. We have used a selective and potent farnesyltransferase inhibitor (FTI) to probe a mechanism of apoptosis that is peculiarly linked to neoplastic transformation. While nontoxic to untransformed mouse cells, FTI triggers a massive RhoB-dependent, p53-independent apoptosis in mouse cells that are neoplastically transformed. Here we offer evidence that the BAR adapter-encoding tumor suppressor gene *Bin1* is required for this transformation-selective death program. Targeted deletion of *Bin1* in primary mouse embryo fibroblasts (MEFs) transformed by E1A+Ras did not affect FTI-induced reversion, actin fiber formation, or growth inhibition, but it abolished FTI-induced apoptosis. The previously defined requirement for RhoB in these effects suggests that Bin1 adapter proteins act downstream or in parallel to RhoB in cell death signaling. The death defect in *Bin1* null cells was significant insofar as it abolished FTI efficacy in tumor xenograft assays. *p53* deletion did not phenocopy the effects of *Bin1* deletion. However, MEFs transformed by SV40 large T antigen+Ras were also resistant to apoptosis by FTI, consistent with other evidence that large T inhibits Bin1-dependent cell death by a p53-independent mechanism. Taken together, the results define a function for *Bin1* in apoptosis that is conditional on transformation stress. This study advances understanding of the functions of BAR adapter proteins, which are poorly understood, by revealing genetic interactions with an Rho small GTPase that functions in stress signaling. The frequent losses of *Bin1* expression that occur in human breast and prostate cancers may promote tumor progression and limit susceptibility to FTI or other therapeutic agents that exploit the heightened sensitivity of neoplastic cells to apoptosis.
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Keywords: ~~Myc; E1A;~~ Ras; RhoB; SV40T antigen; ~~programmed cell death; apoptosis; cancer; transformation;~~ amphiphysin; ~~Bin2; Bin3; Rvs167; Rvs161; cell polarity; cytoskeleton; actin; integrin; NF-kappaB; IkappaB~~

Introduction

Cell suicide processes ~~are thought to~~ play a significant role in ~~limiting~~ cancer ~~progression~~ and therapeutic response. Of particular interest are ~~the~~ processes that are connected specially to the death decisions made by cells ~~clinically~~ harbor preneoplastic or neoplastic lesions. Losses in suicide processes that are germane to cancer pathophysiology would be expected ~~both~~ to promote cancer progression and to blunt therapeutic responses to cancer drugs. While great progress has been made in identifying the basic mechanisms of apoptosis, it remains unclear in many cases exactly how these mechanisms impact cancer and therapy as they occur in humans ~~(in vivo)~~. There has also been little progress is uncovering apoptotic mechanisms that are specially associated with cancer pathophysiology (that is, mechanisms that are germane to cancer as a disease, rather than to apoptosis generally). Such processes are of great interest, as they may help distinguish cancers that will become clinically troublesome and help improve treatment for those that do so.

We have investigated the proapoptotic properties of farnesyltransferase inhibitors (FTIs) as a way to gain novel insight into cancer cell suicide processes. FTIs are a class of experimental chemotherapeutic agents that potently trigger p53-independent apoptosis in transformed ~~mouse or~~ rodent cells, but that often have little to no effect on the survival of nontransformed cells (reviewed in Prendergast, 2000; Cox, 2001). Preclinical tests have established a variety of antineoplastic properties of FTIs in various model systems, including proapoptotic effects; this class of drugs has moved to clinical testing, with ~~perhaps the most~~ promising results emerging recently in breast cancer trials. FTIs were developed originally as a strategy to attack the farnesylation requirement of oncogenic Ras in human cancers.

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*Correspondence: GC Prendergast, Lankenau Institute for Medical Research, 100 Lancaster Avenue, Wynnewood PA 19096, USA; E-mail: prendergastg@mlhs.org

[3]Current address: Johnson and Johnson Pharmaceutical Research Institute, Spring House, PA, USA

[4]Current address: GlaxoSmithKline Pharmaceuticals Co., Inc., King of Prussia, PA, USA

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Recent work, however, has made it clear that FTI responsiveness is based upon factors beyond Ras targeting. Our laboratory has developed a model for the FTI mechanism of action that features the small GTPase RhoB as a critical gain-of-function target of these drugs (Prendergast, 2001). A genetic proof of the crucial and transformation-selective role for RhoB in apoptosis by FTI and other agents used in the clinic to attack cancer cells has been demonstrated recently using a knockout mouse model system (Liu *et al.*, 2000, 2001a, b). Identifying genes that act downstream of RhoB may reveal further insights into this novel cell death mechanism. Loss of such genes would be expected to promote cancer progression and to limit the action of therapeutic agents that exploit the heightened sensitivity of ~~many~~ cancer cells to cell death. This issue is particularly germane to FTI, where clinical studies have not recapitulated the promising results of preclinical testing, perhaps due to secondary events that occur in frank clinical cancers but are absent in model systems.

In this study, we have identified a role for the tumor suppressor gene Bin1 in the transformation-selective ~~process of apoptosis~~ triggered by FTI. Bin1 is an adapter-encoding gene that was initially identified via c-Myc interaction (Sakamuro *et al.*, 1996). Bin1 can induce cell death in human cancer cells and in c-Myc-transformed primary cells (DuHadaway *et al.*, 2001; Elliott *et al.*, 2000). However, the breadth of studies on Bin1 to date suggest a much more complex and integrative signaling function which extends beyond the Myc network. The two ubiquitous Bin1 adapter proteins generated by alternate splicing localize to the nuclear and/or cytosolic compartments of the cell. Bin1 splice isoforms, some of which are alternately termed amphiphysin II isoforms, have been suggested to control actin organization, endocytosis, and transcription (Wigge and McMahon, 1998; Prendergast, 1999). Roles in myoblast differentiation and membrane deformation related to cytosolic localization have been suggested (Wechsler-Reya *et al.*, 1998; Lee *et al.*, 2002). Notably, isoforms of Bin1 that localize to the nucleus can suppress transformation by Myc and other oncogenes, and trigger p53-independent apoptosis in human breast cancers, prostate cancers, melanomas, astrocytomas, and neuroblastomas where expression of the endogenous Bin1 gene is frequently lost, altered, or inactivated (Sakamuro *et al.*, 1996; Elliott *et al.*, 1999, 2000; Galderisi *et al.*, 1999; Ge *et al.*, 1999, 2000a, b; Hogarty *et al.*, 2000; Huang *et al.*, 2000). A recent study of the Bin1 homolog in fission yeast, hob1+, identifies an evolutionarily conserved requirement in stress-signaling and cell cycle control (Routhier *et al.*, 2003). We have proposed that Bin1 adapter proteins act as 'bridging integrators' that link nuclear and cytosolic stress-signaling pathways in cells, perhaps coupling nuclear regulators such as c-Myc to actin control or vesicle dynamics (Prendergast, 1999). Here we show that the role of Bin1 in programmed cell death is contingent on transformation-associated stress. Our results reveal genetic interactions between RhoB and Bin1 in the regulation of cell death in transformed cells. This finding bears on the functions of BAR adapter proteins, which are poorly understood, by supporting the idea that these proteins function in signal transduction. One clinical implication of our work is the potential explanation it offers for why FTI susceptibility is limited in human cancers where Bin1 expression is frequently lost, such as breast ~~and prostate~~ cancers.

Materials and methods

Tissue culture

The generation and characterization of *Bin1* nullizygous animals will be described elsewhere. The heads, limbs, and internal organs were removed from ~~E16.5~~ embryos from pregnant mice. Carcasses were individually minced in DMEM and trypsinized 20 min at 37°C. Fetal bovine serum (FBS) was added to stop the trypsin and the cell suspension was seeded into 25 cm² flasks containing DMEM and 10% calf serum. A portion of this dispersed tissue was used for genotypic analysis. Early passage fibroblasts were derived similarly from embryos from p53 −/− mice (a gift of A Levine). Embryo fibroblasts were transformed at passage 2–3 by transfection with 10 μg each pT22 (mutant human H-Ras vector) and p1A/neo (adenovirus E1A) or pCMVneo Tag (SV40 T antigen) (Prendergast *et al.*, 1991), essentially as described previously (Liu *et al.*, 2000). p1A/neo contains a fragment of the adenovirus genome that expresses the 13S, 12S, and 9S E1A polypeptides. In some experiments, we also examined the response of a mutant H-Ras-transformed mouse embryo fibroblast (MEF) cell clone that arose from a focus but that did not express E1A (presumably established by spontaneous immortalization). Cells were passaged at a 1:5 ratio the day after transfection and transformed cell foci were cloned and expanded into E1A+Ras (ER)- or T+Ras (TR)-transformed cell lines 12–14 days later. The status of Ras farnesylation in ER and TR cells was documented by a Western mobility shift assay that has been described (Liu *et al.*, 2000). A derivative of ER +/+ cells that overexpressed Bcl-2 was generated by transfection of a human Bcl-2 vector (Sakamuro *et al.*, 1995). Transgene expression was confirmed by Western analysis with a Bcl-2 antibody (Santa Cruz Biochemicals).

Cell assays

Cell morphology was documented by photography after 40 h treatment with 10 μM FTI L-744,832 or DMSO carrier (Kohl *et al.*, 1995), or after 24 h treatment with 10 ng/ml TNF-α (Alexis) with or without 2.5 μg/ml cycloheximide (Sigma). Anchorage-independent cell proliferation was monitored in soft agar culture as described previously. The phosphotidylinositol 3′kinase (PI3′K) inhibitor LY294002 (Calbiochem) was used at 10 μM for some apoptosis experiments. For flow cytometry, 5×10^5 cells were seeded onto 60 mm dishes and treated 16 h later with FTI L-744-832 or vehicle in DMEM containing 0.1% calf serum. After 24 h cells were harvested by trypsinization, washed once with PBS, fixed in 70% ethanol, and stained in PBS containing 5 μg/ml propidium iodide, 10 μg/ml RNase A, and 0.1% glucose. Flow cytometry was performed essentially as described previously (Liu *et al.*, 2000), using a FACScan cell analyzer and CellQuest software (Becton-Dickinson). The proportion of cells in the sub-G1 phase DNA fraction, which exhibit DNA degradation, has been demonstrated to be correlated with an apoptotic phenotype in this system (Liu *et al.*, 2000, 2001a).

Gene expression and activity assays

For Northern analysis, total cytoplasmic RNA was isolated from +/+ and −/− MEFs and examined essentially as described (Prendergast and Cole, 1989). Blots were hybridized with a ^{32}P-labeled probe generated from a mouse Bin1 cDNA probe using standard methods. Apoptosis was confirmed in cell extracts by Western analysis of the caspase-3 substrate PARP (Chemicon). Western analysis of other apoptotic proteins in cell extracts was performed using antibodies to TRADD (Santa Cruz Biotechnology), IκB or phosphorylated IκB (Cell Signaling), caspase-3 or caspase-9 (Santa Cruz Biotechnology), cytochrome C (Pharmingen), or Bid (Cell Signaling). Electrophoretic mobility shift assay for NFκB in E1A + Ras transformed MEFs (ER cells) was performed essentially as described (Chen *et al*, 2000). The DNA sequence of the oligonucleotide used for this DNA binding assay was GATGAGTTGAGGGGACTTTCCCAGGC.

Actin immunofluorescence

Cells were seeded onto coverslips in 24-well dishes and treated the next day for 40 h with 10 μM L-744-832 or an equivalent volume of vehicle. Cells were fixed and stained with fluorescein-phalloidin (Molecular Probes) as described previously (Prendergast *et al*., 1994) before analysis and photographed on a Nikon immunofluorescence microscope.

Tumor xenograft assay

ER cells were tested for tumorigenic potential in 6-8 week old scid mice (Fox Chase CB-17 scid mouse strain established at The Wistar Institute by D Bosma). Mice were injected subcutaneously on the upper thigh of different legs of the same animal with 10^6 +/+ or −/− cells suspended in 200 μl of DMEM. Palpable tumors appeared at the injection site within 1 week and visible nodules of >0.5 cm appeared within 2 weeks. For FTI experiments, when the tumor reached ~50–100 mm^3, mice were dosed once daily as described (Kohl *et al*., 1995) by intraperitoneal injection with FTI L-744,832 (40 mpk/day) or 30% DMSO carrier in 0.2 ml total volume. Tumor volumes were calculated using the formula width^2 × length × 0.52.

Results

Farnesyltransferase is susceptible to inhibition in cells lacking Bin1

Experiments in E1A + Ras-transformed [redacted] cells have established that FTIs must elicit a gain-of-function in RhoB to activate apoptosis (Liu *et al*., 2000). However, the elements required downstream or in parallel to RhoB are undefined. p53 mediates many cell suicide events, but p53 is largely dispensable for FTI-induced apoptosis (Lebowitz *et al*., 1997). We therefore explored other tumor suppressor genes that have been implicated in cell death control. Based on previous studies in our laboratory we speculated that FTI-induced apoptosis might involve Bin1, a tumor suppressor gene identified by virtue of its interaction with c-Myc (Sakamuro *et al*., 1996) and implicated recently in programmed cell death processes in human tumor cells and in Myc-transformed primary cells (Elliott *et al*., 2000; DuHadaway *et al*., 2001). To test this hypothesis, we employed a model cell system using embryo fibroblasts that were isolated from mice in which the *Bin1* gene had been targeted for deletion by homologous recombination. Generation and characterization of these mice, which lack expression of all the splice isoforms of the *Bin1* gene, is described elsewhere (Muller *et al*., manuscript submitted).

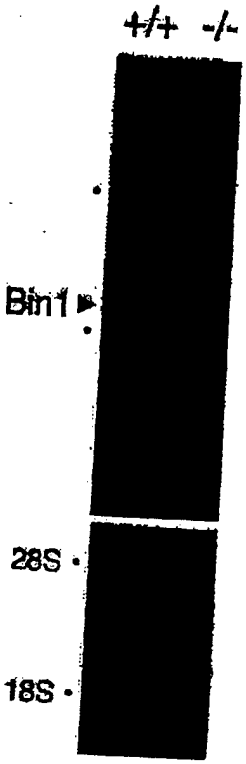

Figure 1 Northern analysis of MEF RNA. Total cytoplasmic RNA isolated from +/+ or −/− primary MEFs were fractionated on Northern gels that were blotted and hybridized to a 32P-labeled *Bin1* cDNA probe. The bottom panel presents a photograph of the ethidium bromide-stained gel as a control for RNA loading

MEFs were isolated from E13.5 embryos that were wild-type (+/+) or homozygous null (−/−) for the *Bin1* gene. Northern analysis confirmed expression of *Bin1* messages in +/+ but not in −/− MEFs, consistent with embryo genotypes (Figure 1). Wild-type MEFs express the two ubiquitous splice isoforms of Bin1; other splice isoforms expressed specifically in the central nervous system or skeletal muscle are undetectable in these cells (Muller *et al*., manuscript submitted). FTIs only activate apoptosis in transformed cells (Lebowitz *et al*., 1997; Suzuki *et al*., 1998), so primary MEFs were cotransformed by adenovirus E1A plus mutant H-Ras. Nullizygous MEFs were susceptible to focus formation and cell lines derived from individual foci readily formed colonies in soft agar (data not shown). E1A + Ras-transformed cells are termed ER cells below and the +/+ or −/− genotype noted refers to *Bin1* unless otherwise indicated. ER cell transgene expression and transformed

phenotype were confirmed by Western analysis and tumor formation in immunocompromised scid mice, respectively (data not shown). We concluded that MEFs lacking *Bin1* are susceptible to oncogene transformation.

Mutant Ras must be farnesylated to cooperate with E1A in primary cell transformation; so Bin1 deletion did not compromise ~~protein~~ farnesylation in cells. To establish that *Bin1* deletion did not affect the susceptibility of endogenous FT to suppression by FTI, for example by promoting drug efflux or turnover, we compared the farnesylation status of H-Ras in drug-treated cells ~~as a pharmacodynamic measurement of FT activity~~. Following 24 h treatment with FTI L-744,832 or vehicle only, ER cells were lysed and processed for Western analysis with anti-Ras antibody Y13-259, as described (Kohl *et al*., 1995). H-Ras farnesylation was blocked with similar efficiency in +/+ and −/− cells, as illustrated by a similar shift in gel mobility that is diagnostic for inhibition of farnesylation (Figure 2). We concluded that loss of *Bin1* does not affect FT activity or susceptibility to inhibition by FTIs.

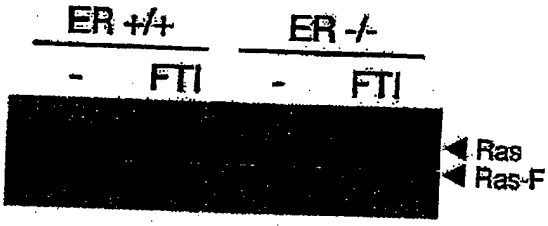

Figure 2 Bin1 deletion does not affect the ability of FTI L-744,832 to inhibit H–Ras farnesylation. H–Ras was used as a pharmacodynamic indicator of FT inhibition. Processing of H–Ras in ER cells treated with vehicle (control) or FTI L-744,832 was monitored as described (Mangues *et al*., 1998), except that anti–Ras monoclonal antibody Y13–259 was used to hybridize the Western blot. The decrease in the apparent gel mobility of Ras is diagnostic for loss of farnesylation and hence inhibition of farnesyltransferase

Bin1 is dispensable for morphological reversion, actin stress fiber formation, and growth inhibition by FTIs

Following FTI treatment, Ras-transformed cells undergo a rapid shift to a flat morphology that is associated with robust actin stress fiber formation (Prendergast *et al*., 1994). We have established that a gain of function alteration of RhoB is sufficient and necessary for these drug responses in MEFs transformed by E1A and Ras (Liu *et al*., 2000). Bin1 adapter proteins are not farnesylated and *Bin1* deletion did not affect the ability of FTIs to elicit the gain-of-function in RhoB, as expected (data not shown). To determine whether Bin1 is essential to various facets of the FTI response, we first compared the morphology and actin stress fiber networks of ER cells treated with FTI. Actin stress fibers were monitored by immunofluorescence microscopy of cells stained with fluorescein-conjugated phalloidin, which binds specifically to filamentous actin. Untreated ER −/− cells displayed a more spindle and rounded morphology and reduced actin ruffles. Nevertheless, these cells exhibited a robust reversion and actin stress fiber response to FTIs in the same manner as ER +/+ cells (Figure 3). Thus, unlike RhoB (Liu *et al*., 2000), *Bin1* was not required for FTI-induced actin reorganization. FTIs also inhibit the proliferation of Ras-transformed cells (reviewed in Prendergast, 2000). A comparison of the proliferation of ER cells in soft agar culture showed that FTIs inhibited colony formation of ER +/+ and ER −/− with the same efficiency (Figure 4). Thus, *Bin1* was also not required for FTI-induced growth inhibition. We concluded that *Bin1* was dispensable for the RhoB-dependent effects of FTIs on ER cell morphology, cytoskeletal actin organization, and anchorage-independent growth.

Bin1 is crucial for apoptosis by FTI

When Ras-transformed cells are deprived of serum growth factors or substratum adhesion, they undergo apoptosis instead of growth inhibition in response to

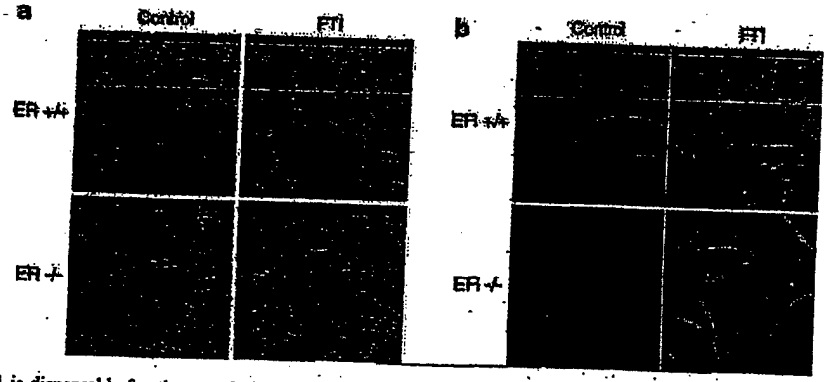

Figure 3 Bin1 is dispensable for the morphological and actin stress fiber responses to FTI treatment. (**a**) Morphology. Cells were photographed 40 h after treatment with 10 μM FTI or vehicle. (**b**) Actin response. Cells were treated with 10 μM FTI or vehicle for 30 h and F-actin was visualized in paraformaldehyde-fixed cells by fluorescein–conjugated phalloidin staining and immunofluorescence microscopy

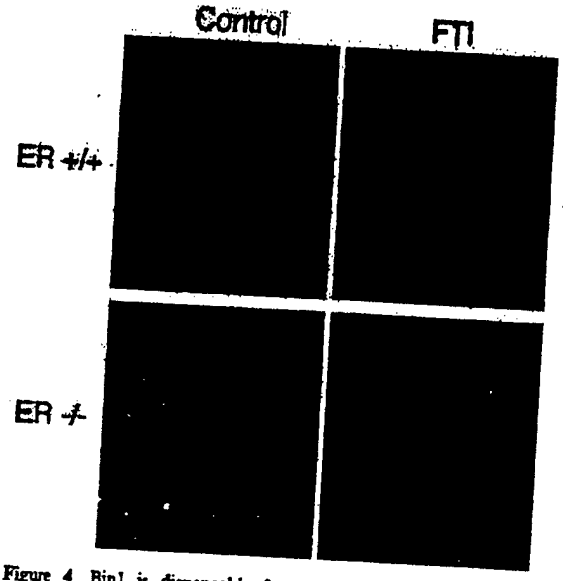

Figure 4 Bin1 is dispensable for suppression of anchorage-independent cell growth by FTI. cells 10^4 were seeded into soft agar culture in the presence or absence of 10 μM FTI and photographed 14 days later

FTI treatment (Lebowitz *et al.*, 1997; Suzuki *et al.*, 1998; Du *et al.*, 1999b; Liu *et al.*, 2000). Which response predominates is determined by the status of the PI3′K–Akt pathway (Du *et al.*, 1999b). Insufficient PI3′K-Akt activity makes cells susceptible to apoptosis. This condition is generated in transformed cells by serum deprival, substratum deprival, or PI3′K inhibition. Under such conditions, RhoB is crucial to engage apoptosis (Liu *et al.*, 2000). To test whether *Bin1* is also crucial, we compared the apoptotic response of the transformed ER +/+ or –/– cells which expressed or lacked Bin1 proteins due to gene deletion.

ER +/+ cells underwent massive apoptosis when exposed to 10 μM FTI under suboptimal serum conditions, as observed previously (Liu *et al.*, 2000), with detached, rounded cells appearing within 16–24 h of drug treatment. Flow cytometry indicated an ~eight-fold increase in the sub-G1 phase population of cells, a diagnostic indicator for FTI-induced apoptosis in this system (Liu *et al.*, 2000, 2001a). In contrast, ER –/– cells underwent morphological reversion to a flat phenotype but did not display increased detachment or rounding. Longer incubation times or higher drug concentrations did not elicit cell demise, arguing that *Bin1* did not merely promote drug response kinetics (data not shown). Flow cytometry confirmed growth inhibition, revealing a reduction of S and G2 phase cells and an increase of G1 phase cells. However, no substantial increase in sub-G1 phase cells occurred. Caspase-3 activation and PARP cleavage occurs during FTI-induced apoptosis (Suzuki *et al.*, 1998; Du *et al.*, 1999b). Characteristic cleavage of PARP occurred in ER +/+ cells, beginning at 4–8 h after FTI treatment, whereas PARP was not cleaved in ER –/– cells (Figure 5b). This response was dependent upon the transformed status of the cell, rather than the presence of E1A, insofar as a similar resistance to FTI-induced apoptosis was displayed by a variant Ras-transformed MEF cell line that arose from a rare focus which did not express E1A (data not shown). Furthermore, the death resistance phenotype was not insurmountable, as treatment with TNF-α plus cycloheximide triggered apoptosis similarly in primary MEFs or E1A+Ras-transformed MEFs that were either wild-type or nullizygous for *Bin1* (data not shown).

To further substantiate the death resistance phenotype of ER –/– cells, we compared the response of ER +/+ cells engineered to overexpress Bcl-2. Western analysis confirmed transgene expression in ER +/+ Bcl-2 cells (Figure 5c, *inset*). The appearance of sub-G1 phase cells was suppressed ~2.5-fold following FTI treatment, as measured by flow cytometry, relative to vector control ER +/+ cells (Figure 5c). The less robust suppression conferred by Bcl-2 in ER +/+ cells supports a different basis for the FTI-resistant phenotype of ER –/– cells. To corroborate these observations, we determined whether ER –/– cells were resistant to apoptosis induced by FTI in the presence of serum plus the PI3′K inhibitor LY294002 (Du *et al.*, 1999b). As observed previously, ER +/+ cells underwent apoptosis when treated 24 h with 10 μM FTI plus 10 μM LY294002 (Figure 6). By itself LY294002 shifted the morphology of ER +/+ cells but did not substantially affect their viability, as quantitated by the proportion of sub-G1 phase cells measured by flow cytometry. In contrast, LY294002 did not cooperate with FTI to activate apoptosis in ER –/– cells (Figure 6). Interestingly, *Bin1* deletion also eliminated the morphological shift caused by LY294002 in the absence of FTI, suggesting that Bin1 may mediate certain PI3′K-dependent effects on cell shape. In summary, these observations supported the conclusion that Bin1 is crucial for apoptosis by FTIs.

p53 deletion does not replicate the apoptotic defect of Bin1 deletion

Although there is evidence that p53 is largely dispensable for the antiproliferative and proapoptotic activities of FTIs (Sepp-Lorenzino *et al.*, 1995; Lebowitz *et al.*, 1997; Barrington *et al.*, 1998), we wished to explicitly address the possibility that *p53* deletion could phenocopy the death resistance displayed by ER –/– cells. Toward this end, *p53* nullizygous MEFs were transformed by E1A plus mutant Ras, generating ER p53 –/– cells for examination (Figure 7). In response to FTI treatment, ER p53 –/– cells underwent morphological reversion, stress fiber induction, and growth inhibition. In suboptimal serum conditions, ER p53 –/– cells also underwent apoptosis (Figure 7, bottom panels). The efficiency of the response as quantitated by flow cytometry was blunted partially relative to ER cells with intact p53. Nevertheless, the result established that *p53* deletion does not abolish FTI-induced apoptosis in the same manner as *Bin1* deletion.

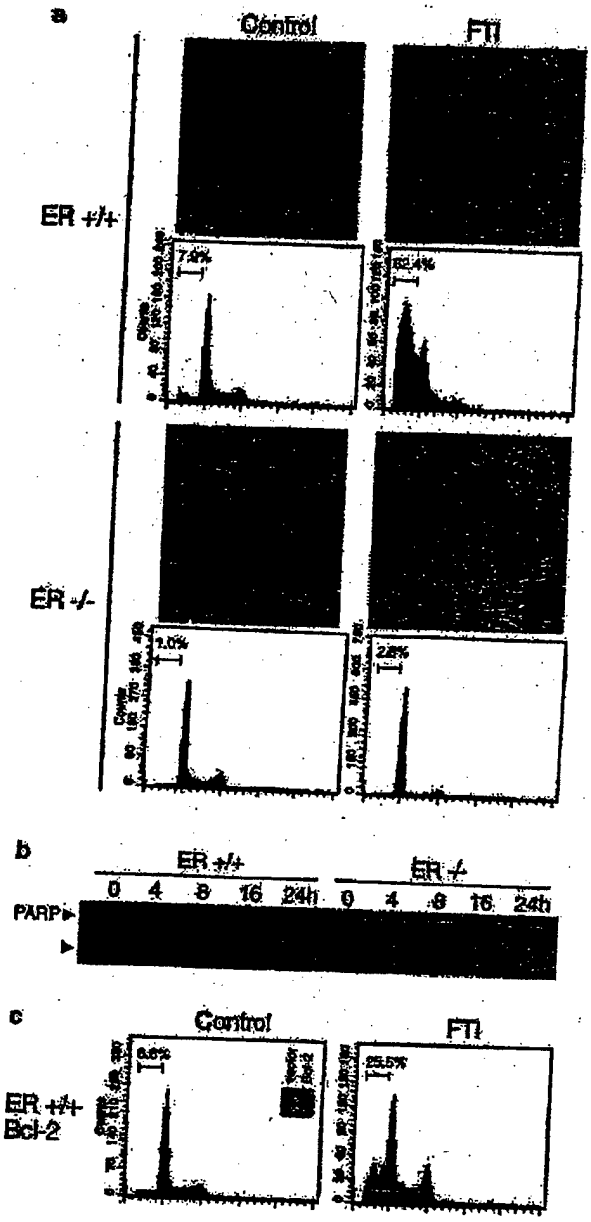

Figure 5 Bin1 deletion abolishes apoptosis by FTI-I. (**a**) Morphology (top panels). ER cells were photographed after 24 h treatment with DMEM containing 0.1% FBS plus either 10 μM FTI or an equivalent volume of DMSO vehicle. Flow cytometry (bottom panels). 5×10^5 cells were seeded into 60 mm dishes, treated as above, and processed as described. The proportion of cells in the sub-G1 phase DNA fraction (degraded DNA) is diagnostic for apoptosis in this cell system (Liu *et al*., 2000). The quantitation of sub-G1 phase cells is noted above the bar in the flow cytometry histograms. (**b**) PARP cleavage. ER cells were treated with 10 μM FTI for the times indicated and cell extracts were prepared and analysed by Western analysis with a PARP antibody. The cleaved product appears below the indicated full-length protein. (**c**) Bcl-2 overexpression does not replicate the Bin1 −/− resistance phenotype. ER +/+ derivatives overexpressing human Bcl-2 (*inset*) were treated as in (**a**)

SV40 T antigen, which inhibits p53-independent cell death induction by Bin1, inhibits apoptosis by FTI

We took a second approach to corroborate the Bin1 requirement in FTI-induced apoptosis, based on evidence that SV40 large T antigen can suppress Bin1-induced cell death in a p53-independent manner (Elliott *et al*., 2000). In these experiments, MEFs were transformed by T antigen plus mutant Ras, generating TR cells for examination. Based on the evidence above, one would predict apoptotic resistance in TR +/+ cells. We confirmed similar susceptibility to FT inhibition in TR cells (Figure 8a). As seen in ER cells, TR cells exhibited similar actin stress fiber and growth inhibitory responses to FTI treatment (Figures 8b and c). In contrast, both +/+ and TR −/− cells were resistant to FTI-induced apoptosis (Figure 8d). This result offered a second line of corroboration for the conclusion that *Bin1* was required for the apoptotic response of transformed cells to FTI treatment.

Bin1 is critical for the antineoplastic response of tumor xenografts to FTI treatment

Previous work established that the apoptotic defect caused by RhoB loss compromises the response of tumor allografts to FTI treatment in vivo. Since *Bin1* deletion produced a similar apoptotic defect, we compared the FTI response of ER +/+ and −/− cells grown as allografts in severe combined immunodeficient (scid) mice. Cells (10^7) were injected into the opposite thighs of the same animal, to control for nonspecific environmental effects, in a total of twenty mice. Palpable +/+ and −/− tumors in each animal formed within 7 days of injection. At 1 week after the allograft was initiated, mice were assigned randomly to control or drug treatment groups. The drug treatment group was dosed once daily for 16 days by intraperitoneal injection with 40 mg/kg FTI L-744,832 as described (Kohl *et al*., 1995). Control mice were given vehicle carrier only. Tumor volumes were calculated from caliper measurements taken at various times during the trial. No adverse side-effects of L-744,832 administration were observed consistent with previous observations (Kohl *et al*., 1995; Barrington *et al*., 1998; Mangues *et al*., 1998; Liu *et al*., 2000). At the end of the trial, mice were euthanized and their tumors were surgically excised for weighing and examination.

ER allografts exhibited a significantly different susceptibility to FTI suppression (Figure 9). −/− tumors exhibited a higher apparent growth rate, but the difference with +/+ xenografts was not statistically significant. Similar to previous observations (Liu *et al*., 2000), +/+ tumors were strongly inhibited by the drug protocol and grew little relative to controls. In contrast, −/− tumors continued to grow robustly such that by the end of the experiment they were statistically similar to controls in size. The fact that −/− tumors continued to grow in FTI-treated animals implies that apoptotic capacity plays a predominant role in the in vivo

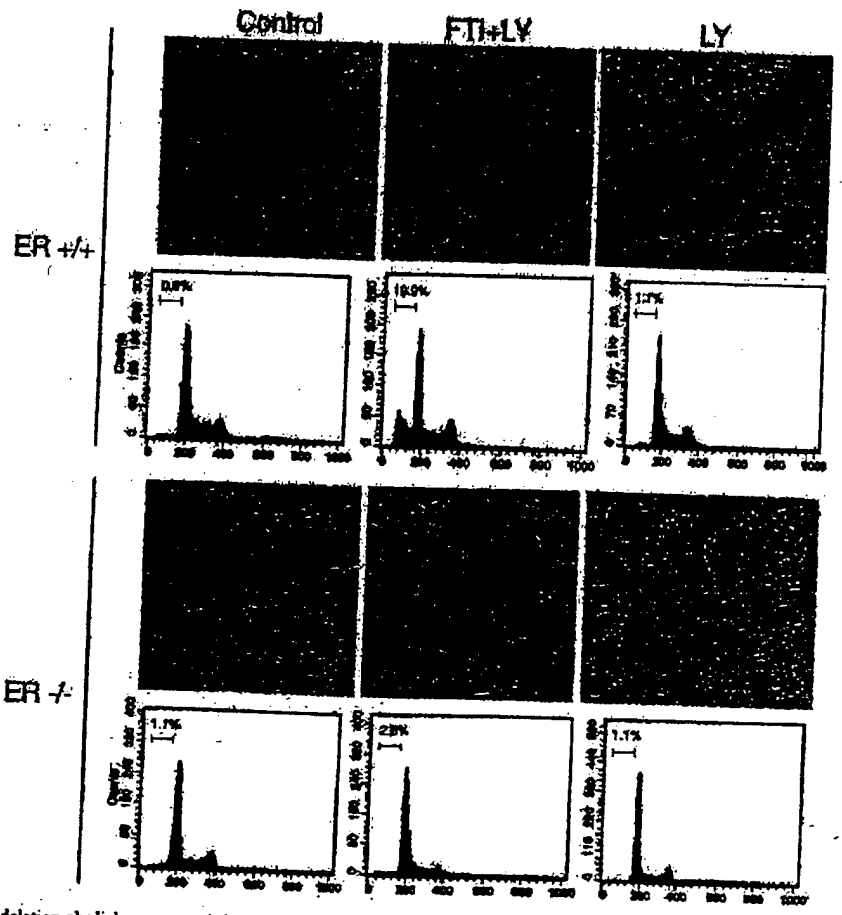

Figure 6 Bin1 deletion abolishes apoptosis by FTI-II. Morphology (*top panels*). ER cells were photographed after 24 h treatment with DMEM containing 10% FBS plus DMSO vehicle or 10 μM FTI in the presence or absence of 10 μM L294002. Flow cytometry (*bottom panels*). Cells were treated as above and processed as before. The quantitation of sub-G1 phase cells is noted above the bar in the flow cytometry traces

response. We concluded that Bin1 is crucial to the antineoplastic response of tumor cells to FTI treatment.

Discussion

This study defines a critical role for the tumor suppressor Bin1 in the cell suicide program engaged by FTI in transformed primary cells. This program is marked by its peculiar selectivity in transformed cells: most FTIs studied to date have little to no effect on the viability of nontransformed mouse cells, but they are potent inducers of apoptosis in cells that are transformed by Ras and certain other oncogenes. This characteristic makes FTIs a useful probe of cell death processes that are intimately connected to the pathophysiology of neoplastically transformed cells. While FTIs have a variety of effects on transformed mouse cells, such as reversion, stress fiber induction, and growth inhibition, we demonstrated that *Bin1* is required only for FTIs to trigger apoptosis. The apoptotic defect in cells lacking *Bin1* is significant because the defect compromised the antitumor efficacy of FTIs in vivo. Bin1 proteins appear to act downstream or in parallel to the FTI target protein RhoB, which is sufficient and/or necessary to mediate the major features of the FTI response in the mouse, including apoptosis, via a gain-of-function mechanism (Du *et al.*, 1999a; Du and Prendergast, 1999; Liu *et al.*, 2000). These findings identify Bin1 as a second element with RhoB that is part of a cell death process linked to transformed cell pathophysiology. RhoB has important functions in stress signaling (Prendergast, 2001) and recent genetic investigations in fission yeast support a similar role in stress signaling for *Bin1* (Routhier *et al.*, 2003). Thus, the specific requirements for RhoB and Bin1 in FTI-induced apoptosis may reflect broader roles for these genes in a unique stress signaling process. Preliminary experiments using TNF-α, another factor that selectively targets the survival of transformed cells, support the conclusion that *Bin1* has a function in apoptosis that is contingent upon a transformation-related stress. Loss of Bin1 compromised the ability of TNF to trigger

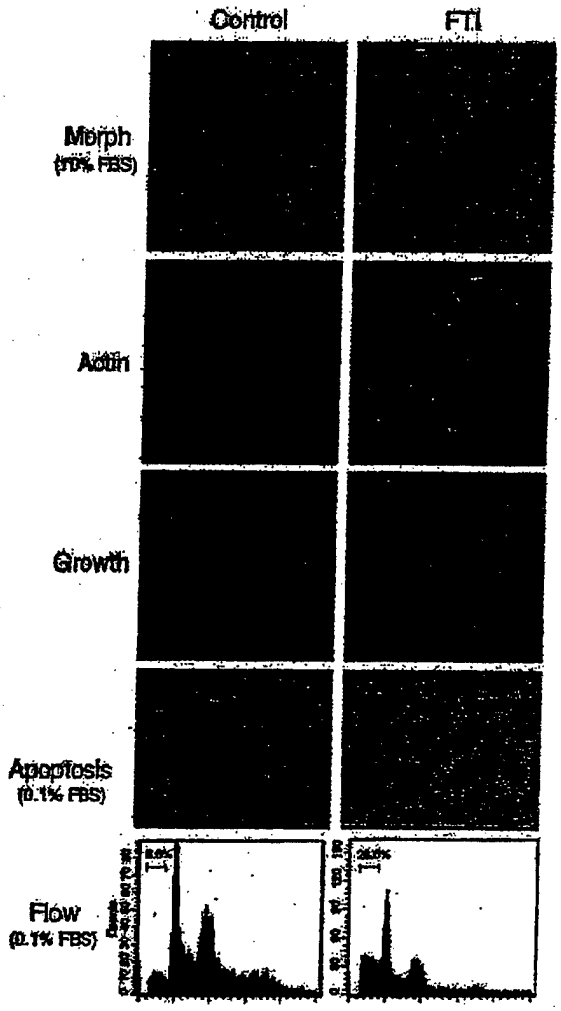

Figure 7 p53 deletion does not replicate the Bin1 defect. ER p53 −/− cells were exposed to 10 μM FTI or DMSO vehicle in DMEM containing 10% FBS (*morph, actin, growth panels*). Morphology was documented by photography 40 h after FTI treatment. Actin and cell growth in soft agar culture were assayed as above. Apoptotic response was assessed in DMEM containing 0.1% FBS. After culturing 24 h in the presence of 10 μM FTI or DMSO vehicle, cells were photographed and the harvested and processed for flow cytometry as above. The quantitation of sub-G1 phase cells is noted above the bar in the flow cytometry traces

apoptosis in ER cells, but it did not affect the ability of TNF plus cycloheximide to kill primary cells or ER −/− cells. In this cell system, the death defect compromised mitochondrial release of cytochrome *c* and activation of caspases-9 and -3 in a manner that was associated with dysfunctional control of NFkB, at a level beyond IkB regulation (unpublished observations). Taken together, these observations argue that Bin1 is needed to mediate the death sensitization caused by neoplastic transformation in certain settings.

During cancer progression, a strong selection ~~would~~ be expected against transformation-associated 'death penalties' that limit cell survival. Therefore, the functional status of genes that mediate such processes would be expected to have a significant impact on cancer progression. Such genes would also be expected to impact therapeutic responses, in cases where the 'therapeutic window' is based upon heightened death sensitivity. The results of the present study suggest that *Bin1* status may be an important determinant of apoptotic susceptibility to FTI. Notably, losses of *Bin1* expression occur frequently in advanced breast and prostate cancers (Ge *et al*., 2000a, b) and *Bin1* is frequently inactivated by missplicing during the progression of malignant melanoma (Ge *et al*., 1999). These observations may be relevant to the interpretation of ongoing FTI clinical trials which aim at assessing and interpreting efficacy in various cancers, including the ones mentioned above.

Bin1 has been implicated in a variety of processes but its function has yet to clearly emerge. This gene encodes several alternately spliced adapter proteins, the first of which was identified by virtue of its functional interaction with c-Myc (Sakamuro *et al*., 1996; Elliott *et al*., 1999). Recent studies support a role for *Bin1* in death signaling in c-Myc-transformed cells and in human cancer cells (Elliott *et al*., 2000; DuHadaway *et al*., 2001), where *Bin1* is frequently ~~downregulated~~ or inactivated by missplicing (Ge *et al*., 1999, 2000a, b). In these studies, *Bin1* was implicated in cell death in malignant or transformed primary cells, but not in nonmalignant or nontransformed cells. The present study supports and extends the earlier work, by using a knockout system that can offer more robust and direct support of a role for *Bin1* in cell death signaling.

While initial studies focused on proposed connections between Bin1 and c-Myc, the breadth of work to date on the *Bin1* gene reveals a more complex and integrative signaling function that extends beyond the Myc network. Bin1 proteins are members of the BAR family of adapter proteins, including the budding yeast Rvs proteins and the mammalian amphiphysin protein, which have been implicated in actin regulation, endocytosis, and nuclear processes (Wigge and McMahon, 1998; Colwill *et al*., 1999; Bon *et al*., 2000). Budding yeast studies in particular suggest an important role for the actin cytoskeleton as a key site of action for BAR adapter proteins (Breton and Aigle, 1998; Balguerie *et al*., 1999; Colwill *et al*., 1999). However, Bin1 appears to have functions that differ from and/or extend beyond those defined for amphiphysin and the Rvs proteins, as suggested for example by (i) the nuclear localization of certain Bin1 isoforms and their functional interaction with nuclear Myc and Abl proteins (Kadlec and Pendergast, 1997; Wechsler-Reya *et al*., 1997; Elliott *et al*., 1999), (ii) the lack of endocytosis defects in *Bin1* −/− cells (A Muller *et al*., manuscript submitted), and (iii) the ability of Bin1 to complement defects in stress-induced cell cycle control ~~rather than actin organization or endocytosis~~ that are generated in fission yeast following deletion of the *Bin1* homolog *hob1+* (Routhier *et al*., 2003). In summary, Bin1 adapter proteins

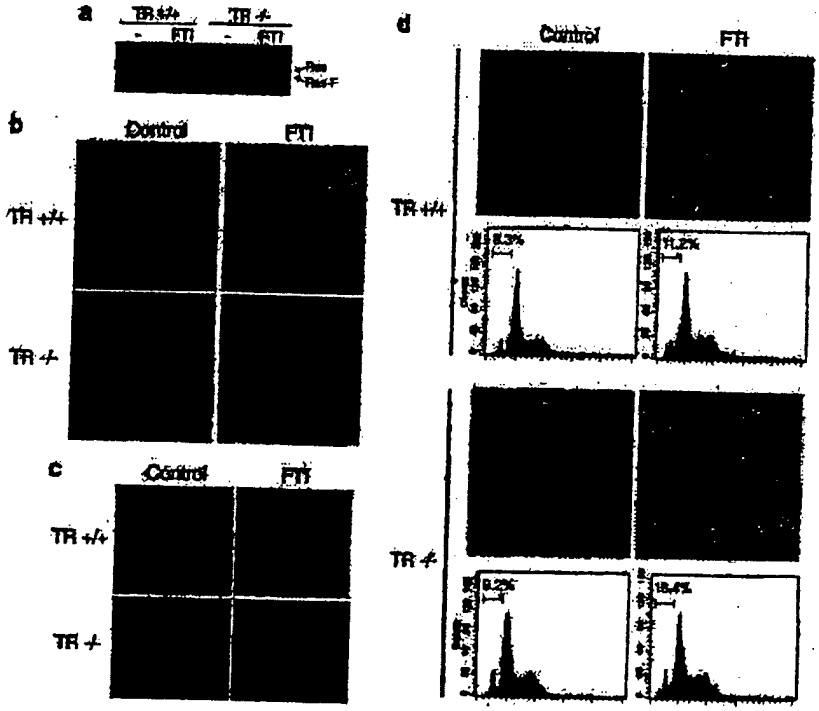

Figure 8 SV40T antigen suppresses FTI-induced apoptosis. (**a**) Susceptibility of FT to inhibition. The farnesylation status of H-Ras in TR cells was monitored by Western analysis as in Figure 2. (**b**) Actin response. TR cells were treated and processed for actin staining as above. (**c**) Growth inhibition. TR cells 10^4 were seeded into soft agar culture in the presence or absence of 10 μM FTI and photographed 14 days later. (**d**) Apoptosis. Morphology (*top panels*) of TR cells was documented as before after culturing 24 h in DMEM plus 0.1% FBS in DMSO vehicle or 10 μM FTI. Flow cytometry (bottom panels) was performed as above. The quantitation of sub-G1 phase cells is noted above the bar in the flow cytometry traces

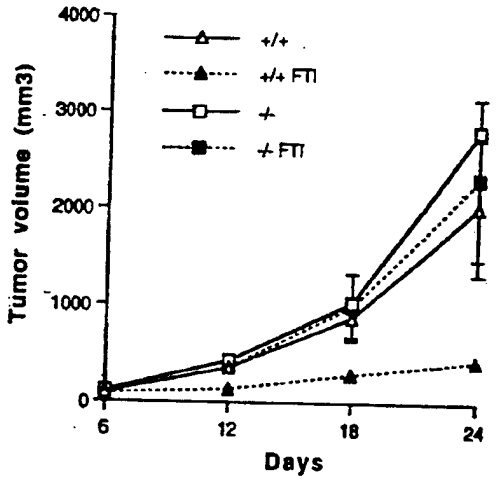

Figure 9 Bin1 deletion compromises the anti-tumor efficacy of FTIs 10^7 ER +/+ or −/− cells were injected subcutaneously into opposite thighs of a single scid mouse (total of 20 mice). Mice were assigned randomly to drug treatment or control groups and 1 wk later dosed daily for 16 days by intraperitoneal injection with FTI (40 mg/kg) or vehicle carrier. One animal in the treatment group died as a result of an errant injection. Tumor size was measured by caliper and tumor volume was computed as described in the Materials and Methods. The data represent the average and standard mean error of the tumor volume as computed at the times indicated

have complex signaling functions in animal cells, perhaps related to stress signaling, that have yet to be fully understood.

Other connections between RhoB and Bin1 proteins can be drawn in support of their involvement in a common signaling process. Bin1 proteins lack evident Rho-interaction motifs and do not appear to associate with Rho proteins. However, a Rho guanine nucleotide exchange factor (RhoGEF) was recently identified as a Bin1-binding protein in our laboratory, and several other members of the human BAR adapter family actually include RhoGEF and RhoGAP domains as part of their structure (AM and GCP, unpublished observations). Moreover, some of these genes include membrane-targeting sequences such as pX and PH domains that in some cases have been linked to actin-based systems or vesicle dynamics. Neuronal isoforms of Bin1, also referred to as amphiphysin II, are found in specialized endocytotic complexes that function in synaptic vesicle endocytosis (Wigge and McMahon, 1998). While studies in *Bin1* knockout mice have failed to identify an essential role for this gene in endocytotic processes (Muller *et al*., manuscript submitted), the presence of at least some Bin1 isoforms in endocytotic complexes suggests some role in those complexes, perhaps to integrate stress signals. Genetic analysis of RVS167, the ortholog of *Bin1* in budding yeast, points to direct interactions with actin (Bauer *et al*., 1993;

Amberg *et al.*, 1995; Breton and Aigle, 1998; Balguerie *et al.*, 1999), including a role in coordinating cytoskeletal and cell cycle responses (Lee *et al.*, 1998). Recent work on *hob1+*, the homolog of *Bin1* in fission yeast, suggests that the function of *Bin1* may have diverged to some extent during evolution. Fission yeast cells lacking *hob1+* do not display actin cytoskeletal or endocytotic defects, like budding yeast cells lacking RVS167, but instead display defects in stress responses triggered by starvation or DNA damage (Routhier *et al.*, 2003).

The likely intersections of Bin1 with stress signaling, actin, and membrane dynamics are intriguing in light of the same intersections made by RhoB, which is localized in cytosolic membranes but ~~perhaps~~ also to the nucleus and/or nuclear membrane (Adamson *et al.*, 1992; Lebowitz *et al.*, 1995; Zalcman *et al.*, 1995; Lebowitz and Prendergast, 1998; Michaelson *et al.*, 2001) (L Benjamin, pers. comm.). In the endosomal compartment, RhoB regulates intracellular receptor trafficking through interactions with its effector kinase PRK (Mellor *et al.*, 1998; Gampel *et al.*, 1999), which has also been implicated in apoptosis (Cryns *et al.*, 1997; Takahashi *et al.*, 1998; Koh *et al.*, 2000). Given the possibility of an effector role for Bin1 in the FTI cell suicide program, it is tempting to speculate that Bin1 may be regulated or modified at some level by Rho signals that are actin-based but directed to or from the nucleus. Regulation by PI3′K signaling may also be germane, given that Bin1 deletion compromises the morphological as well as the proapoptotic effects of the PI3′K inhibitor LY294002 in the transformed MEF system. ~~Lastly, although needing further assessment, a possible intersection with NFκB regulation is consistent with links between RhoB and NFκB regulation that have been documented recently (Fritz and Kaina, 2001) and with a nuclear function for some Bin1 isoforms, perhaps in transcriptional control (Elliott *et al.*, 1999).~~ This study offers a biological foundation for investigations of the genetic interactions between RhoB and Bin1 and of a unique death-signaling mechanism that links these genes to transformed cell stress and cancer pathophysiology.

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